Preparing Entomocidal Products with
Oligosporogenic Mutants of
'Bacillus thuringiensis'

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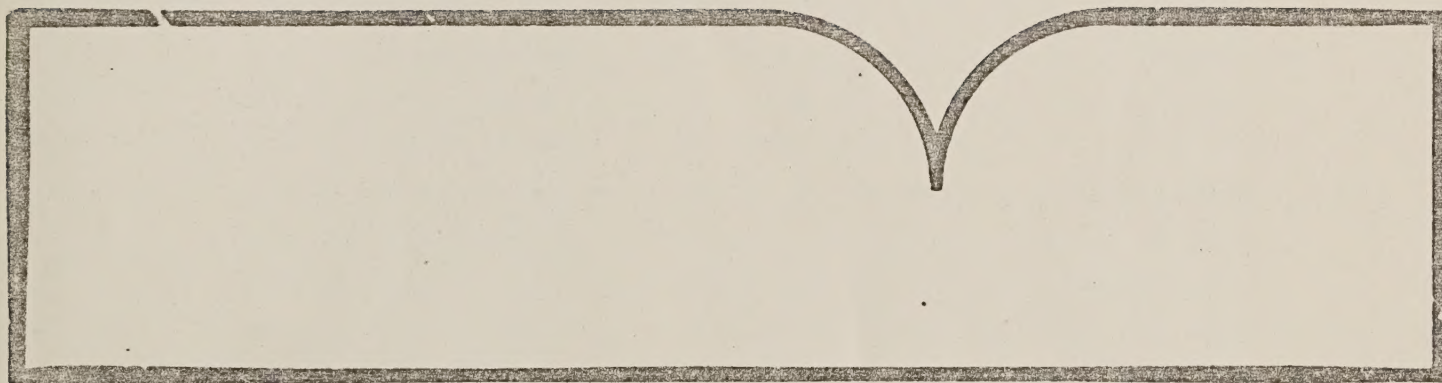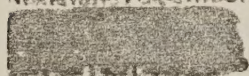
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Preparing Entomocidal Products with
Oligosporogenic Mutants of Bacillus thuringiensis

Abstract of the Disclosure

Six oligosporogenic mutant strains obtained from Bacillus thuringiensis subsp. kurstaki yield parasporal products substantially free of interfering spores when cultivated under normal sporulation conditions. These parasporal products have utility in the biological control of pest insects.

Background of the Invention

Field of the Invention

With the recent trend toward biological pest control, considerable attention has been given to the insecticidal properties of the bacterium Bacillus thuringiensis. Of particular interest is the subspecies, kurstaki, because of its broad and effective entomocidal host range. The subspecies is commercially prominent for biological control of lepidopteran pest insects.

The sporulating cells of B. thuringiensis each produce a spore (endospore) and a diamond-shaped proteinaceous crystal (paraspore or inclusion body). The entomocidal properties have been attributed solely to the δ-endotoxin which is a major component of the parasporal crystal. When the crystal solubilizes in the insect gut, it gives rise to a protoxin which is activated by proteolytic digestion.

At the completion of sporulation, the autolyzing cells release both the spores and the crystals into the culture medium. For most investigative research purposes, it is desirable that the latter be recovered in a substantially pure state. Also, environmental considerations dictate that commercial insecticidal preparations be substantially free of viable spores. However, the similarity in size and density renders separation of the crystals and spores both complicated and laborious. This invention relates to a method for the production of

parasporal products which simplifies, or even obviates, such a
separation.

Description of the Prior Art

It had been known from the teachings of Shieh et al., U.S. Patent
No. 3,758,383, that it was possible to select a mutant strain of B.
thuringiensis which could be cultured under sporulation conditions
without the usual production of the crystal bodies. Attempts at the
reverse situation have led to reports of other mutants which fail to
sporulate but which do form the crystalline paraspores. Nishiitsutsuji-
Uwo et al. [J. Invert. Path. 25: 355-361 (1975)] shows the selection
of five completely asporogenous strains from mutagenized wild types.
Two of these were derived from the subspecies kurstaki, but overall
were somewhat less toxic than the parent strain. Other attempts to
mutate B. thuringiensis have led to strains which produce neither
spores nor crystals [A. A. Yousten, Can. J. Microbiol. 24: 492-494
(1978)].

Summary of the Invention

I have now discovered six oligosporogenic mutant strains of B.
thuringiensis, subspecies kurstaki, capable of producing parasporal
products which are substantially free of contaminating spores and
which yield activated protein having toxigenic activity equal to or
greater than that of the parent strain.

In accordance with this discovery, it is an object of the
invention to select and isolate the novel strains pursuant to muta-
genesis of the wild type.

It is also an object of the invention to cultivate the mutant
strains on a nutritionally sufficient sporulation medium in order
to induce the formation of parasporal crystals.

A further object of the invention is to recover substantially
spore-free entomocidal products from the media of the cultivated
strains.

Other objects and advantages of this invention will become
readily apparent from the ensuing description.

Detailed Description of the Invention

The novel mutant microorganisms of this invention have been derived from a single parent strain of B. thuringiensis subsp. kurstaki NRRL B-3792 (HD-1) according to a procedure adapted from the method of Adelburg et al. [Biochem. Biophys. Res. Commun. 18: 788-795 (1965)] for mutating Escherichia coli. The mutagen used in this procedure is N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which typically induces death to approximately 95-98% of the cultured bacteria. Upon plating, a percentage of the survivors produce colonies with translucent or less pigmented sectors. From such sectors, oligosporogenic mutants that retain the ability to form parasporal crystals are isolated and then streaked until pure. The terms "oligosporogenic" and "oligo-sporogenous" are used herein in reference to the condition of producing an abnormally small number of spores. Selection of the instant mutants in accordance with this procedure is described in detail in Examples 1 and 2 below.

EXAMPLE 1

B. thuringiensis subsp. kurstaki NRRL B-3792 (HD-1) was cultured overnight at 30° C. on NSMP medium having the following composition:

	grams/liter
Casamino acids (Difco)	5.0
glucose	2.0
KH_2PO_4	0.932
K_2HPO_4	0.549
sodium citrate	0.588
$MgCl_2 \cdot 6H_2O$	0.2033
$CaCl_2 \cdot 2H_2O$	0.1029
$MnCl_2 \cdot 4H_2O$	0.0099
$ZnCl_2$	0.00068
$FeCl_3$	0.00016
distilled H_2O	1000 ml.
pH	6.5

The culture was then reinoculated onto fresh NSMP medium and incubated
at 30° C. for 4 hours. After the incubation, the cells in their
exponential phase of growth were washed aseptically by centrifugation
twice with saline (0.87% NaCl) and resuspended in 10 ml. saline in a
5   sterile 50-ml. flask. Ten milliliters of 0.2% solution of MNNG were
added to the flask for a final concentration of 1 mg. MNNG/ml. This
resulted in an approximate kill rate of 99.9%. After incubation on
a water bath shaker for 30 minutes at 30° C., the suspension was
washed by centrifugation twice with sterile saline and resuspended
10   on 50 ml. of fresh NSMP growth media. At the end of 4 hours of
incubation, the culture was serially diluted in 0.1% tryptose broth
dilution tubes and 0.1 ml. was spread onto NSMP agar plates which were
incubated for 2-3 days at 30° C. Colonies were screened for sporeless
mutations by microscopic examination and visual selection of lighter,
15   less-pigmented sectors. Three oligosporogenous, crystal-producing
isolates (1A1, 1A2, and 1A3) were selected and each was restreaked
until pure and stable.

<center>EXAMPLE 2</center>

The mutagenesis procedure of Example 1 was repeated except that
20   10 ml. of 0.02% MNNG was used for a final broth concentration of
100 µg./ml. and the kill rate was approximately 99.6%. Three
oligosporogenous, crystal-producing isolates (2A71, 2A72a, and
2A72b) were selected and each was restreaked until pure.

The mutant strains isolated from Examples 1 and 2 above have
25   been deposited in the Agricultural Research Culture Collection (NRRL)
in Peoria, Illinois, and have been assigned the following accession
numbers:

1A3	NRRL B-4453
2A72a	NRRL B-4454
1A1	NRRL B-4455
1A2	NRRL B-4456
2A71	NRRL B-4457
2A72b	NRRL B-4458

While individual taxonomic characterizations were not developed
for each of these strains, it is presumed that they possess substantially
the same characteristics as the wild type, with the exception of their
sporulation capacity. All six isolates are similar in morphological
appearance when viewed with a phase microscope. Refractile spores
are not visible, but indications of aborted sporulation are apparent
in the form of phase-darkened bodies in polar regions and asymmetrical
septum formation. All isolates produce large crystals, occurring
either singularly or in multiples per cell, and as in the parent
strain, they oftentimes are somewhat asymmetrical in shape.

Electron microscopy of thin sections taken from 24-hour-old
preparations reveals that all six isolates are blocked at stage II of
sporulation or before. In many instances, no spore-related structures
are observed; in others, a sporulation septum is visible; and in some,
complete engulfment of the forespore has occurred. Visible evidence
of cortex synthesis is lacking. Parasporal crystals are generally
tetrahedral in shape, but fractures and surface irregularities are
often visible. Thin sections of free crystals are characterized by
numerous surface irregularities and protrusions, much like the embedded
bodies described by Sharpe and Baker [J. Invertebr. Path., in press
(1980)]. Removal of the embedded body leaves concavities or depressions
in the crystalline surface. Repeating regularly spaced lattice fringes
at the edges of the crystal, indicative of the internal hexagonal
array of crystalline subunits that comprise the fully formed paraspore,
are also apparent in thin-section high-magnification micrographs.

In the production of a parasporal product by any of the oligo-
sporogenic mutants, any known solid or liquid sporulation medium
normally used for producing spores and crystals by cultivating the
wild-type B. thuringiensis may be employed. The NSMP medium described
above for use in the mutagenesis procedure is well suited for this
purpose. Of course it is understood that the production of spores
will not be positively influenced by the medium beyond the sporulation
capability of the mutant cells.

The cultivation conditions are typical of those employed for
sporulation of the wild type. Thus, for optimum production, temperatures
will be on the order of 30° C. and incubation periods will be from
about 2 to about 5 days. However, since viability of the isolates

5   diminishes rapidly after 8 hours, maintenance cultures must be
incubated no longer than 8-10 hours and stored immediately thereafter
for preservation.

The crystals may be recovered from the culture medium by any
conventional procedure known in the art. Washing with centrifugation

10 separates the crystals and cellular debris from the liquid components
of the medium resulting in a crude parasporal product. Since the
mutant strains are characterized by a low sporulation rate, this
crude product will be substantially free of spores suitable for most
commercial applications. If desired, the cellular debris can be

15 easily removed using buoyant density centrifugation. For a more
refined preparation, the solids may be resuspended in 1 M NaCl and
held at about 37° C. until the nucleoprotein derivatives are denatured.
After additional washing, final isolation of a pure parasporal fraction
completely devoid of spores is readily accomplished by linear gradient

20 centrifugation. The initially reduced number of spores eliminates
overloading of the gradients with its consequent overlapping of the
spore and crystal bands.

The percent sporulation for cultures of the novel mutant strains
is typically between about 0.01% and 0.13% without significant loss

25 of crystal yield. Therefore, the spore-crystal ratios are in the
range of from about 1:800 to about 1:8000. For purposes of this
invention, crystal preparations having a spore count within this
range are considered to be substantially spore-free. Actual spore
counts are illustrated in Example 3 below.

30   EXAMPLE 3

B. thuringiensis subsp. kurstaki parent strain NRRL B-3792 and
the six mutant strains obtained in Examples 1 and 2 were cultivated on
NSMP medium in 50-ml. shake flasks at 30° C. for 24 hours. After
samples from each culture were plated for viable counts of vegetative

cells, aliquots of the cultures were heated at 80° C. for 12-15
minutes to kill the existing vegetative cells. Samples were then
plated to ascertain the spore count of each culture. The results
are shown in Table I below.

Table I

Strain (NRRL)	Viable count (vegetative cells/ml. culture)	Spore count (spores/ml. culture)	% Sporulation
B-3792 (parent)	8×10^7	7.5×10^7	93.8
B-4453	6.8×10^6	4.5×10^3	0.066
B-4454	1.6×10^7	2.1×10^3	0.013
B-4455	8×10^6	5×10^3	0.063
B-4456	6×10^6	$7.4 \times 10^-$	0.123
B-4457	5×10^6	1×10^3	0.020
B-4458	8×10^6	4.3×10^3	0.054

5   The toxicity of the parasporal proteins isolated from the
various mutant strains appears to be comparable to or better than
that of the wild-type δ-endotoxin. This fact has been established
by data from in vitro bioassays conducted in accordance with the
procedure set forth in Example 4, below.

10   EXAMPLE 4
        A. Preparations containing crystals and cellular debris resulting
from growth of the parent and the six isolate strains of Examples 1 and
2 on agar plates or in shake flasks were washed twice by centrifugation
with 10 ml. tris (hydroxy-methyl) aminomethane (Tris), pH 7.8, and

15   0.05 M KCl. Washed pellets were resuspended in 1 M NaCl and heated
at 37° C. for 18 to 24 hours to hasten the removal of nucleoprotein
derivatives from the vegetative debris. The suspensions were then
centrifuged and the pellets were given four more washes in saline
solution at 37° C. The resulting pellets were then resuspended in a

20   small volume of 1 M potassium thiocyanate (KSCN) and placed upon a

linear gradient of 40-80% sodium diatriazoate (either "Hypaque
Sodium" or "Renografin"). The gradients were centrifuged at 8000
x $\underline{g}$. for 2 hours. The crystals concentrated in a band two-thirds of
the way down the tube, with most cellular debris retained diffusely
5   throughout the upper part of the gradient. Crystals were collected,
washed in buffer, and reseparated on fresh gradients for a total of
three passages through sodium diatriazoate. The resulting crystal
preparations were free of contamination from spores or cellular
debris.

10   B. To prepare the purified crystals for bioassay, the para-
sporal protein was first solubilized by suspension in 0.2 $\underline{M}$ sodium
thioglycollate in 0.1 $\underline{M}$ sodium bicarbonate buffer, pH 9.5, at 30° C.
for 2 hours. The resultant protein solution, or protoxin, was
dialyzed overnight against 10 mM Tris, pH 8.5, 1 mM sodium thio-
15   glycollate, and 1 $\underline{M}$ KSCN. Solubilized parasporal protein solutions
were adjusted to an approximate protein concentration of 5 mg./ml.
and activated by enzymatic digestion with α-chymotrypsinogen A
(bovine pancreas) at a level of 200 µg. enzyme/ml. of soluble
parasporal protein. Millimolar quantities of Ca^{++} and Mg^{++} were
20   added to the solubilized mixture to aid enzymatic digestion, which
was conducted at 32° C. for 4 hours in a reciprocating water bath.

        C. Bioassays were performed by adding 0.1 ml. of the activated
parasporal protoxin solution to 0.1 ml. of buffered insect saline
(BIS) containing 1.5 to 2.0 x 10^5 FPMI-CF-1 cells from minced neonate
25   larvae of Choristoneura fumiferana (spruce budworm) and incubating
for 30 minutes at 28° C. The incubation was terminated by the
addition of 2 ml. of boiling 0.05 $\underline{M}$ Tris buffer, pH 7.7, to each
assay tube and boiling for an additional 10 minutes. Residual
adenosine triphosphate (ATP) was measured with a Packard Tri-Carb
30   liquid scintillation spectrometer by the luciferin-luciferase assay
as described by Stanley and Williams [Anal. Biochem. 29: 381-392
(1969)]. This assay measures the ATP content of cultured insect
cells after treatment with toxin. Undamaged cells retain normal
levels of ATP, but affected cells lose ATP to the culture milieu.

Hydrolytic enzymes such as ATPase lost from the same damaged cells quickly destroy free ATP. In practice, the maximum lethal response of cultured insect cells to parasporal protein averages 70-80% of the total cell population. Consequently, the decrease in assayed

5   cell culture ATP, corrected by the method of Murphy [Science 194: 954-956 (1976) for the number of resistant cells, is a measure of the amount of toxin added to the culture. LD_{50} values (the dose lethal to 50% of the susceptible cells) were determined by this technique and are reported in Table II below.

<div align="center">

Table II

Strain (NRRL)	LD_{50} [a]
B-3792 (parent)	13.4
B-4453	10.3
B-4454	4.5
B-4455	10.5
B-4456	8.1
B-4457	7.0
B-4458	6.3

</div>

a   µg. parasporal protein
    per ml. digest (containing
    $1-2 \times 10^5$ cultured insect
    cells)

10   It is understood that the foregoing detailed description is given merely by way of illustration and that modification and variations may be made therein without departing from the spirit and scope of the invention.
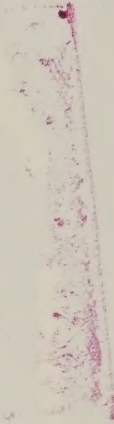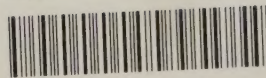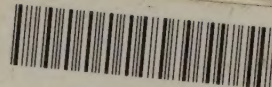
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